DESCRIPTION

A METHOD FOR CONTINUOUS CULTURE OF THE ANAEROBIC MICROORGANISMS

Technical Field

The present invention relates to a method for continuous culture of anaerobic microorganisms.

Background Art

Among anaerobic microorganisms, there are many industrially important bacteria such as acetone-butanol producing microogranisms, lactic acid producers, and ethanol producing bacteria. However, a process to culture these microorganisms for modern industrial application is not known except for the conventional batch culture. Acetone-butanol fermentation was the first of the most popular modern fermentation processes until the World War II, however, acetone and butanol are now produced by the petrochemical process and no country is using the former process.

It is well known that lactic acid bacteria are useful for the food industry, not only dairy products but also conventional pickling fermentation. Lactic acid production as an organic acid production process is still in the development stage unlike glutamic acid fermentation which is the most modern aerobic industrial fermentation among the amino acid and nucleic acid fermentations.

It is known that ethanol fermentation by bacteria has some advantages over the yeast process, but no satisfactory process has been introduced in the ethanol industry. Nevertheless many studies involving continuous reactors using immobilized cell process have been made.

Current issues such as alternatives to fossil fuel, reduction of carbon dioxide emission, and environmental hazards by plastic waste, have resulted in attention to the efficient culture methods of anaerobic microorganisms. Lactic acid fermentation in large scale mass production to supply raw material for biodegradable polylactic acid synthesis, and efficient ethanol fermentation process to supply economically sufficient ethanol for gasohol is being paid great attention on an urgent basis. However, a continuous fermentation process for lactic acid fermentation using *Lactococcus* and *Lactobacillus* and ethanol fermentation using *Zymomonas* that are possible to apply industrially have not been developed.

There are many scientific papers describing continuous fermentation of anaerobic microorganisms, but these reports relate to chemostat culture without proper control of the fermentation kinetics so that continuous fermentation can not last for a long time with adequate control of all fermentation dynamics, resulting in uneconomical residual glucose concentration with large substrate loss. Thus, these results are not available for an industrial fermentation process.

The continuous fermentation process which is industrially available must exhibit a long stationary fermentation state with stable kinetics. The feed rate is maintained constant and residual substrate concentration is as low as possible to minimizeloss of the raw material. In continuous fermentation employing aerobic fermentation, DO-stat is often used.

In aerobic fermentation, as substrate concentration approaches the critical value (lower limit), cell activity decelerates to raise dissolved oxygen level due to decrease of oxygen uptake rate of the cells. Substrate is then fed and fermentation activity will be recovered promptly to last the process.

Anaerobic microorganisms do not require oxygen for their metabolism and DO-stat is not possible to use as a substrate feed.

In another aerobic fermentation, pH-auxostat which is the method for substrate feed using pH rise when the substrate is used up, is employed. Likely in anaerobic fermentation, pH turning to up from down is often observed in ethanol formation as well as lactic acid formation, and pH-auxostat may be introduced for anaerobic continuous fermentation. In practice, pH-auxostat in aerobic fermentation was a failure. In anaerobic microorganisms, cells lose their activities irreversibly when pH changes to rise.

As shown in Fig. 1, anaerobic fermentation is possible to last by pH change caused by alternative addition of substrate and alkaline (A) and substrate is fed before residual substrate concentration approached the critical value (low limit) (B). On the contrary, as shown in Fig. 2, fermentation ceases if substrate is not fed when pH turns to rise (A) and residual substrate concentration became lower than the critical value (B). These are special characteristics of anaerobic microorganisms unlike characteristics of aerobic microorganisms. Therefore a method has been found for substrate feeding for anaerobic continuous fermentation.

Another problem to solve for continuous anaerobic fermentation is low cell population due to sterile cell formation that is defined by the inventor. Sterile cells are often found in anaerobic bacterium and such cells cannot make daughter cells so that such cell growth remains at a low level of maximum growth. To increase fermentation rate, high cell population is the effective means and such culture system can be prepared by cell recycling culture. Cell recycling brings high cell population and at the

same time serious end product inhibition due to increase of product concentration in the culture broth. Thus cell recycling culture results in serious end product inhibition resulting in unstable fermentation kinetics and very poor productivity.

The present invention is directed to developing new control methods for anaerobic continuous fermentation that is available for industry such as high purity L-lactic acid production and biofuel ethanol production. These processes require very high economical efficiency to minimize production costs.

Disclosure of Invention

To overcome technical problems mentioned above, the present invention provides following solutions:

1. A method for continuous culture of the anaerobic microorganisms where active cell population is maintained constant, when the fermentation is operating continuously by feeding substrate and alkaline solution alternatively, residual glucose concentration of the culture liquid can be controlled by feeding the substrate at a rate is equal to the alkaline consumption rate.

2. The method for continuous culture of the anaerobic microorganisms according to an above-mentioned invention, wherein the residual glucose concentration is maintained constant by feeding substrate of molarity that is equal to cumulative consumption molarity of alkaline solution added in order to control pH of the culture liquid.

3. The method for continuous culture of the anaerobic microorganisms according to either of above-

mentioned inventions, wherein the using diluted alkaline solution forms large dilution effect of culture liquid resulting high specific activity of the microorganisms and high volumetric productivity are maintained.

The present invention has been completed by the following studiesconducted by the inventor.

The direct control of residual glucose concentration in the culture liquid by feed back control of substrate feed was not successful. The suitable sensor and other digital signals to represent the substrate consumption could not be found so that it is found that the required amount of glucose supply can be calculated by the amount of alkaline solution consumed as long as cell activity is maintained. In this system, residual glucose concentration can be controlled at a pre-set level. In this fermentation system, high cell density culture gives high volumetric productivity; however, such culture conditions make high product concentration thereby developing strong product inhibition. This results in deceleration of the specific rate of the fermentation dynamics.

Control of residual glucose concentration of such fermentation is difficult. By introducing turbidity control to this fermentation system, cell population comes under control. This fermentation system makes high product concentration by high cell density but high productivity is still obtained by using diluted alkaline solution to obtain a large dilution effect. In this manner, end product inhibition in high cell density culture becomes smaller and such fermentation becomes stable with fairly good residual glucose concentration.

Thus the present invention has been completed.

Using the present invention, continuous culture of anaerobic microorganism has succeeded

with fairly good low glucose concentration in the harvest. This process can be applied to industrial production of L-lactic acid. The products from this process are low in cost and of very high quality with low remaining glucose in the final products.

Brief Description of Drawings

Fig. 1 shows that in the culture of anaerobic microorganisms, as pH of the culture liquid approaches a lower preset limit (A), at that time residual glucose concentration decreases to the critical value. Fermentation proceeds effectively, when residual glucose concentration remains above the critical level (B);

Fig. 2 shows that fermentation will cease and can not recover if substrate will be fed after residual glucose concentration become lower than the critical value;

Fig. 3 shows a schematic diagram of the mechanical set-up and control system for the present invention.

Best Mode for Carrying Out the Invention

In the present invention, cell density of anaerobic microorganism is controlled and continuous culture is conducted by alternative feed of substrate and alkaline solution. Residual glucose concentration of the spent medium is controlled by the alkaline feed rate using the equation for calculation based on molar equivalent of glucose consumption rate and correction factor. By this control system, raw material loss in the spent medium decreased so that the process can produce high

quality product with the minimum cost performance.

Also in the present invention, the active cell population is increased by recycling the cells to increase the product concentration resulting strong product inhibition. The productivity of the process can be recovered to high level by high dilution effect performance for the culture system employing diluted alkaline solution for feeding. By these means, specific activity of the fermentation can be maintained high and anaerobic continuous fermentation become stable for long term operation.

The operation of the system is explained using a schematic diagram. In Fig. 3., the principle structure of this fermentation system is depicted by a SFD (Schematic Flow Diagram). In this figure, A: fermentor, B: cross flow filtration for cell separation, C: pH indicator and controller, D: computer for calculation of feed rates of the substrate based on alkaline consumption rate, E: turbidity controller, using laser probe, P1, P2, and P3: peristaltic pumps are indicated. And *F*1: rate of alkaline flow-in to operate pH-stat culture, *F*2: rate of substrate flow-in determined from alkaline consumption rate, *F*3: rate of the medium that does not contain the glucose added for turbidity control flow-in, *F*4 and *F*5: cell free broth exit for feed back control of *F*1 and *F*2 respectively to control fermentation working volume constant, *F*6: broth exit, for feed back control of *F*3 to control the working volume constant.

All rates are expressed in a dimension of ml/min. To maintain constant working volume (V) of the fermentor A, the relationship of *F*1=*F*4 and *F*2=*F*5 should be retained and at the same time to maintain cell population constant under constant working volume the relationship of *F*3=*F*6 must be preserved. In this culture system, sum of the rates of stream into the fermentor is *F*1+*F*2+*F*3 and sum of bleeding out from the fermentor is *F*4+*F*5+*F*6. To maintain constant working volume, the

relationship of $F1+F2+F3=F4+F5+F6$ should be established. Thus unlike the conventional reasoning for continuous culture, dilution rate of this continuous fermentation is expressed by the following equation (1).

$$D = \frac{F1 + F2 + F3}{V} = \frac{F4 + F5 + F6}{V} \quad (1)$$

To constant cell population in the fermentor, total growth of the cell in unit time, μXV should be equal to cell bleeding out, $XF6$, so that specific growth rate of this fermentation system can be expressed by the relationship $XF6=\mu XV$;.

$$\mu = \frac{F6}{V} \quad (2)$$

The conventional reasoning for continuous culture has been expressed by Monod equation which defines that continuous culture will attain the steady state at operation condition of $\mu=D$. The present invention does not agree with this theory so that this is a new type continuous fermentation that is controlled by new logic. In this new control system, substrate feed rate is given by the function of alkaline feed rate $F1$. If alkaline concentration is diluted to low normality, culture broth will be diluted to increase productivity due to the dilution effect. In this way, end product inhibition becomes smaller and the specific activity of the cells is maintained high so that fermentation becomes steady with fairly good remaining glucose concentration.

The following section, describes the examples of the present invention. And of course, the present invention is not limited by the following examples.

Examples

Example 1:

The strain used is *Lactococcus lactis* IO-1 (JCM7638) which was isolated by the inventor was used as a stock culture stored in a deep freezer at -85 °C. It was refreshed in TGC liquid medium (Difco Laboratories, Detroit) and transplanted into 100 ml medium containing in an Erlenmeyer flask for 8 h culture. The medium consisted of 3% of glucose, 0.5% of yeast extract, 0.5% of poly-peptone and 1% g of NaCl and autoclaved for 5 min at 120 °C. The fermentation system employed is the same one shown in Fig. 3. The fermentor is a glassware 1 liter jar with an inner agitation rd driven by magnetic force of gentle agitation 400 rpm. The jar was put in a water bath to which 37 °C water was being circulated. A glass electrode for pH measurement (Toa Denpa Go. Tokyo) was installed in the jar and pH of the culture liquid was controlled at the lower limit value (pH 6.0) by feeding of alkaline solution (1N-NaOH). Residual glucose level was periodically determined by an enzymatic glucose analyzer and when glucose concentration reached to 3 g/l continuous culture was started by feeding of substrate the rate of which was calculated based on the rate of alkaline consumption rate. The relationship between glucose demand and alkaline consumption can be considered as the following. That is, the glucose quantity (G_Q) can be written by the following equation (3),

$$G_Q = \frac{fF_1 \times 90}{0.95} + C \quad (3)$$

where f is a coefficient of normality of 1N-NaOH and C is a term for adjustment of the residual glucose concentration. Glucose demand (g) is equivalent to lactic acid (M.W.=90) formation (g) which corresponds to the rate of 1N alkaline flow-in (F_1), however 5 % of the fed glucose should be lost for regeneration of the microorganisms. Therefore, the glucose demand is divided by 0.95. Off-set of the control system can be adjusted by the term C according to monitoring residual glucose concentration.

Therefore, to supply a glucose quantity which is calculated by an equation (3) using the glucose concentration S g/l, medium feed rate can be calculated by,

$$F_1 = \frac{G_Q}{S} \quad (4)$$

where S is glucose concentration (g/l) of feed solution.

Continuous culture was conducted by feeding the substrate solution according to the equation (3) and (4). Cell free filtrate from the cross flow ultra filtration was withdrawn The culture broth was withdrawn and recycled back to the fermentor through a cross flow ultra filter (MICROZA PSP103, Asahi-kasei Co., Tokyo). Permeate of the filtration was harvested as lactic acid product solution. Turbidity of the culture broth was determined and controlled at constant cell density by bleeding out the culture broth and feeding non-glucose nutrition solution (yeast extract 0.5%, poly-peptone 0.5% and

NaCl 1.0%) as a diluent using a DDC controller (Model LA-300 ASR Co., Tokyo). All streams of the feeding in and bled out were synchronized by a peristaltic pump. Three kinds of feed solutions (*F*1, *F*2 and *F*3) were fed in and two kinds of solution (*F*4 and *F*5, and *F*6) were bled out. Substrate supply rate is calculated from the alkaline consumption rate for neutralizing lactic acid.

After 12 h batchwise cultivation, residual glucose concentration reached 3 g/l and the fermentation turned to continuous culture. With substrate feed and cell recycling, cell concentration gradually increased to about 10.5 g/l.

Residual glucose concentration in broth was 4.5 g/l. which is higher than the target value of 2.0 g/l. However, glucose concentration was reduced by manipulating the term *C* of the equation (3). For 3 h operation, residual glucose concentration has approached to 2±0.5g/1. Continuous operation lasted for 10 days, 250 h with total dilution rate 0.7 1/h. During continuous operation, average lactic acid concentration of the harvest was 45 g/l, therefore the volumetric productivity of this fermentation was 31.5 g/lh.

From the harvested liquid, L-lactic acid was purified and concentrated to 90 %. Remaining glucose in the product was less than 5 % against L-lactic acid and it is of satisfactory quality for poly-lactic acid synthesis.

Example 2:

The strain used is *Lactococcus lactis* IO-1 (JCM7638) which was isolated by the inventor was used a stock culture stored in a deep freezer at -85 °C which was refreshed in TGC liquid medium (Difco Laboratories, Detroit) and transplanted into 100 ml medium containing in an Erlenmeyer flask

for 8 h culture. The medium consisted of 3% of glucose, 0.5% of yeast extract, 0.5% of poly-peptone and 1% of NaCl and autoclaved for 5 min at 120 °C. The fermentation system employed is the same one used in the Example 1. The fermentor is a glassware 1-liter jar with an inner agitation rd driven by magnetic force of gentle agitation at 400 rpm. The jar was put in a water bath to which 37 °C water was being circulated. A glass electrode for pH measurement (Toa Denpa Go. Tokyo) was installed in the jar and pH of the culture liquid was controlled at the lower limit value (pH 6.0) by feeding of alkaline solution (1N-NaOH). The liquid employed for fermentation was enzymatically-hydrolyzed cornstarch diluted to 5 % glucose equivalent. Enzymes employed were NOVO Thermamyl 120L and Dextrozyme 225/75 L. To the syrup, 1% of corn steep liquor (CSL) was added and pH of the liquid was adjusted before autoclaving at 120 °C for 5 min.

Residual glucose level was periodically determined by an enzymatic glucose analyzer and when glucose concentration reached to 1.5 g/l, continuous culture was started by feeding of substrate, the rate of which was calculated based on the rate of alkaline consumption rate. The relationship between glucose demand and alkaline consumption was given by the equation used in Example 1.

These feeding rates were controlled by the computer. The culture liquid was withdrawn and recycled back to the fermentor through a cross flow ultra filter (MICROZA PSP103, Asahi-kasei Co., Tokyo) and cell free filtrate from the cross flow ultra filtration was withdrawn as a harvest solution to recover lactic acid product. To the fermentor, a laser working probe for turbidity determination was installed to control the cell density of the culture broth. The cell density of the culture broth was controlled at constant by bleeding out the culture broth and feeding non-glucose nutrition solution

(CSL 1.0%) as a diluent, using a DDC controller (Model LA-300 ASR Co., Tokyo). All streams of the feeding in and bleeding out were synchronized by a peristaltic pump. Three kinds of the feed solutions (*F*1, *F*2, and *F*3) were fed in and two kinds of harvest solution (*F*4 and *F*5, and *F*6) were bled out. Substrate which is glucose supply rate is calculated from the estimated rate of addition of alkaline solution (0.5N-NaOH) for control of pH.

After 18 h batchwise cultivation, residual glucose concentration approached 1.5 g/l and the fermentation shifted to continuous culture. With substrate feed and cell recycling, cell concentration gradually increased to about 12.3 g/l of DCW.

Residual glucose concentration in culture liquid was 1 g/l. that were lower than the target value of 1.5 g/l. However, glucose concentration was increased by manipulating the term *C*. After 1 h operation, residual glucose concentration has been controlled to 1.5±0.2 g/1. Continuous operation lasted for 3 weeks, 525 h with total dilution rate 1.0 1/h. During continuous operation, average lactic acid concentration of the harvest was 40.5 g/l, therefore the volumetric productivity of this fermentation was 40.5 g/lh.

From the harvested liquid, L-lactic acid was purified and concentrated to 90 %. Remaining glucose in the product was less than 5 % against L-lactic acid and it is of satisfactory quality for poly-lactic acid synthesis.

Example 3:

Microorganism, *Zymomonas mobilis* NRRL-B14023, was used. Stock culture stored in a deep freezer at -85°C was refreshed in YM liquid medium (Difco Laboratories, Detroit) and

transplanted into 100 ml medium containing in an Erlenmeyer flask for 8 h culture. The medium consisted of 100 g of glucose, 10 g of yeast extract, 1 g of KH_2PO_4, 1 g of $(NH_4)_2P0_4$ and 0.5 g of $MgSO_4$. $7H_20$ in one liter of deionized water and autoclaved for 5 min at 120 °C. Continuous culture was conducted in the fermentation system demonstrated in Fig. 3.

The fermentor is a glassware 1 liter jar with an inner agitator driven by magnetic force of 400 rpm of gentle agitation. The jar was put in a water bath to which 37 °C water was being circulated. A glass electrode for pH (Toa Denpa Go. Tokyo) was installed in the jar and pH of the culture broth was controlled at two pre set points, an upper limit and lower limit, using a pH controller. In continuous culture, substrate was fed at upper pH limit and alkaline solution (0.5N-NaOH) was fed at the pH lower limit. The culture broth was withdrawn and recycled back to the fermentor through a cross flow ultra filter (MICROZA PSP103, Asahi-kasei Co., Tokyo). Permeate of the filtration was harvested as ethanol product solution. Turbidity of the culture broth was determined and controlled at constant cell density by bleeding out the culture broth and feeding non-glucose nutrition solution as a diluent using a DDC controller (Model LA-300 ASR Co., Tokyo). All streams of the feeding in and bleeding out were synchronized by a peristaltic pump. Substrate supply is calculated from the alkaline consumption for glucose intake by the following equation (5),

$$G_Q = \frac{fF1\, f_H \times 180}{0.95} + C \quad (5)$$

where f_H indicates a reciprocal number of ml of 1N-NaOH required for 1 mole (180 g) of glucose

intake. Stoichiometry of ethanol fermentation from glucose shows one mole of carbon dioxide release for one mole formation of ethanol. Thus unlike lactic acid fermentation, ethanol yields from glucose theoretically 50 %, not 100%.

Then, if the rate of conversion of ethanol from glucose is expressed as f_H, an equation (5) will be given by the same as an equation (1).

In order to maintain constant working volume, an accurate volume of cell free filtrate must be withdrawn when substrate solution and alkaline solution was flown in. In the same way, glucose free diluent was fed when the culture broth is bled out to reduce cell density, so that the working volume of the continuous culture is not changed.

The medium consisted of 10% of glucose, 1% of yeast extract, 0.5% of KH_2P0_4, 0.1% of $(NH_4)_2S0_4$ and 0.05 % of $Mg(S0_4) \cdot 7H_20$. A 20 ml of the seed culture was transferred to 400 ml of working volume for start up of the culture. pH of the culture was maintained at pH5.5 by feeding alkaline.

At 8 h after start of the main fermentation, residual glucose concentration reached 1 g/l and continuous culture was introduced with cell recycling. Cell concentration increased and glucose feeding rate proportionally increased. Then the cell concentration reached 7.5g/l as DCW. Residual glucose concentration in the broth was slightly high e.g. 1.5 g/l. However, glucose concentration was adjusted by manipulating the term *C*. For 2 h operation, residual glucose concentration has been maintained 1.0±0.3g/1. Continuous culture lasted for 7 days of 150 h with total dilution rate of 0.5 1/h and satisfactory low residual glucose level. Ethanol concentration of the harvested liquid was 52 g/1 so

that ethanol productivity of this continuous culture was 26 g/l h.

Industrial Applicability

In the present invention, microorganisms employed are not immobilized and maintain cell activity at a healthy state at while cells which have lost activity and sterile cells are allowed to washout from the reactor and to regenerate the healthy cells. Thus this reactor will consist of the active and healthy cells. Such bioreactor gives a small deviation of the fermentation kinetics by introducing turbidity control for cell population. The system can easily operate in a continuous mode by feeding substrate and bleeding out the culture broth. Dilution rate of this system is given by the total volume of the feeding solution including alkalinesolution. Diluted alkaline solution makes a large dilution effect, resulting in small end product inhibition. When cell density become high, product concentration increases and represses the product formation rate. However in such case, product formation rate can be maintained high by use of diluted alkaline solution. This type of bioreactor is unknown. The rate of the bioprocess reaction of this new reactor is large as in a petrochemical process in continuous operation, so that volumetric productivity of this bioprocess gives a few tens of times of that of batchwise operation.